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(54) Title: IMMORTALIZED HUMAN CELL LINES CONTAINING EXOGENOUS CYTOCHROME P450 GENES

(57) Abstract

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Non-tumorigenic, stable, human bronchial and liver epithelial cell lines are provided wherein the cell lines are capable of expressing human cytochrome P450 genes which have been inserted into the cell lines. Also provided are methods and kits for identifying potential mutagens, cytotoxins, carcinogens, chemotherapeutic and chemo-preventive agents utilizing these cell lines.

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IMMORTALIZED HUMAN CELL LINES CONTAINING EXOGENOUS CYTOCHROME P450 GENES

BACKGROUND OF THE INVENTION

The invention is related to immortalized human bronchial epithelial cells and human liver epithelial cells containing various cytochrome P450 genes and the uses of these cells. The invention is also related to the construction and application of recombinant vectors containing DNA sequences for encoding, and efficient expression of, enzymatically active cytochromes P450 in mammalian cells.

The cytochromes P450 are a large family of hemoprotein enzymes capable of metabolizing xenobiotics such as drugs, carcinogens and environmental pollutants as well as endobiotics such as steroids, fatty acids and prostaglandins. Some members of the cytochrome P450 family are inducible in both animals and cultured cells, while other constitutive forms are non-inducible. This group of enzymes has both harmful and beneficial activities. The harmful activity is the metabolic conversion of xenobiotics to toxic, mutagenic and carcinogenic

forms. The beneficial activity is the detoxification of xenobiotics (Gelboin, Physiol. Rev., 60:1107-1166, 1980).

In animals, multiple molecular forms of cytochrome P450s are expressed simultaneously and they all exhibit common physical and biological properties. The multiplicity and common properties of cytochromes P450 make it difficult to separate their different forms, especially the minor forms. Even in situations where P450 cytochromes have been isolated in purified form by conventional enzyme purification procedures, they have been removed from the natural biological membrane association and therefore require the addition of NADPH-cytochrome P450 reductase and other cell fractions for enzymatic activity. These additional factors have prevented a clearer understanding of the role and function of the individual cytochrome forms in metabolism, detoxification, and activation of both xenobiotic and endobiotic substrates.

Toxicological testing of drugs, potential carcinogens, food products, food additives and food contaminants has been performed in animals and more recently in in vitro systems, such as bacteria (Ames test) and animal cell culture models. These systems are disadvantaged since they do not have human-specific metabolism. Therefore, extrapolation to determine the human risk is difficult and potentially inaccurate. The bacterial test systems and some of the animal cell culture models lack complete metabolic activity and would not detect any harmful compounds which depend upon activation by metabolic pathways, for example, by the cytochrome P450 enzymes. In the past this situation was circumvented by adding metabolizing enzyme isolated from rat livers to the cultured animal cells. This approach poses two significant problems. First, the resulting metabolism is not necessarily the same as in man. Secondly, highly-reactive metabolites might not reach their target molecule and, consequently, escape detection.

Although human metabolizing enzymes have been introduced into a human cell line, this system suffers from serious deficiencies. (Crespi, Progress in Clinical and Biological Research, Vol. 340B Mendelsohn and Albertini (eds)

Wiley-Liss, New York 97-106, 1990.) The human cells are lymphoblasts which do not constitute a major target tissue of cytotoxins, mutagens, or carcinogens and have no natural cytochrome P450 activity in the absence of inducers. In addition, other enzymes involved in the activation process, for example, epoxide hydrolase, are missing in these cells and must be introduced by gene transfer methodology. This system therefore comprises an artificial model with a questionable correlation to the in vivo situation.

SUMMARY OF THE INVENTION

Therefore, it is desirable to have an in vitro human cell line system which parallels the in vivo human condition. The present invention provides isolated non-tumorigenic human cell lines of bronchial and liver epithelial cell origin with unlimited proliferative potential, resulting in immortalization.

In one embodiment of this invention a non-tumorigenic, stable, human bronchial epithelial cell line is provided wherein the cell line is capable of growing without senescence when cultured in vitro in growth medium and contains an exogenous cytochrome P450 gene which is capable of being expressed in the cell line. The gene can be inserted by transfection or infection. P450 genes expressed in this cell line include 1A1, 1A2, 2A6, 3A3, 3A4, 2B6, 2B7, 2C9, 2D6, and/or 2E1. Preferred cell lines include any one of cell lines BEAS-2B-1A1, BEAS-2B-1A2, BEAS-2B-2A6, BEAS-2B-3A3, BEAS-2B-3A4, BEAS-2B-2B6, BEAS-2B-2B7, BEAS-2B-2C9, BEAS-2B-2D6, BEAS-2B-2E1 or a homolog or a derivative of these cell lines. The BEAS-2B-1A1 cell line is a BEAS-2B cell line containing the cytochrome P450 1A1 gene, the BEAS-2B-1A2 cell line is a BEAS-2B cell line containing the cytochrome P450 1A2 gene, and so forth. P450 genes are preferably operably linked to a cytomegalovirus promoter to obtain efficient expression. A particularly preferred cell line is BEAS-2B-1A2 or a homolog or derivative thereof.

In a second embodiment of this invention a non-tumorigenic, stable, human liver epithelial cell line is provided wherein the cell line is capable of growing without senescence when cultured in vitro in growth medium and contains an exogenous cytochrome P450 gene capable of being expressed in the cell line. The gene can be inserted by transfection or infection. P450 genes expressed in this cell line include 1A1, 1A2, 2A6, 3A3, 3A4, 2B6, 2B7, 2C9, 2D6, and/or 2E1. Preferred cell lines include any one of cell lines THLE-5B-1A1, THLE-5B-1A2, THLE-5B-2A6, THLE-B5-3A3, THLE-5B-3A4, THLE-5B-2B6, THLE-5B-2B7, THLE-5B-2C9, THLE-5B-2D6, THLE-5B-2E1 or a homolog or derivative of these cell lines. The THLE-5B-1A1 cell line is a THLE-5B cell line containing the cytochrome P450 1A1 gene, the THLE-5B-1A2 cell lines is a THLE-5B cell line containing the cytochrome P450 1A2 gene, and so forth. P450 genes are preferably operably linked to a cytomegalovirus promoter to obtain efficient expression. A particularly preferred cell line is THLE-5B-1A2 or a homolog or derivative thereof.

In another embodiment of this invention, various methods of utilizing the cell lines are described. For example, a method for identifying or testing the mutagenicity, cytotoxicity, or carcinogenicity of an agent is described which comprises the steps of: a) reacting, culturing, or contacting the cell line with an agent suspected of being a mutagen, cytotoxin, or carcinogen, and b) determining or monitoring those effects on, or changes in, the cell line which are indicative of mutagenicity, cytotoxicity, or carcinogenicity.

Also described by this invention is a method for identifying or testing the chemotherapeutic or chemopreventive activity of an agent comprising the steps of: a) reacting, culturing, or contacting the cell line with an agent suspected of being a chemotherapeutic or chemopreventive in the presence of a carcinogen, and b) determining or monitoring those effects on, or changes in, the cell line which are indicative of chemotherapeutic activity. The agent can be added prior to the carcinogen to measure the preventative effects of the agent.

In a further aspect of this invention, a method is provided for determining the metabolites activated by a carcinogen or xenobiotic comprising the steps of: a) reacting, culturing or contacting the cell line with the suspected carcinogen or xenobiotic, and b) identifying the metabolites and/or their effects.

Also provided are diagnostic kits comprising the cell lines, media, and reagents for use in one of the methods.

Various other objects and advantages of the present invention will become apparent from the Detailed Description of the Invention.

BRIEF DESCRIPTION OF THE DRAWINGS

These and other objects, features and many of the attendant advantages of the invention will be better understood upon a reading of the following detailed description when considered in connection with the accompanying drawings wherein:

Figure 1 shows the schematic construction of the recombinant vectors for expressing cytochrome P450 genes and the transfection of the vectors into the BEAS-2B cells.

Figure 2 shows a map of the pCMV and cytochrome P450 fragments for insertion into this vector.

Figure 3 shows a Western blot of BEAS-2B-CMV-1A2 cells confirming expression of CYP1A2 in these cells. Abbreviations: B=BEAS-2B, cl=clone, m=microsomal.

Figure 4 shows a Western blot of BEAS-2B-CMV-2A6 cells confirming expression of CYP2A6 in these cells.

Figure 5 shows a Western blot of BEAS-2B-CMV-3A4 cells confirming expression of CYP3A4 in these cells.

Figure 6 shows a Western blot of BEAS-2B-CMV-2E1 cells confirming expression of CYP2E1 in these cells.

Figure 7 shows a Western blot of BEAS-2B-CMV-2D6 cells confirming expression of CYP2D6 in these cells.

Figure 8 shows a Western blot of THLE-5B-CMV-1A2 cells confirming expression of CYP1A2 in these cells. Abbreviations: T=THLE-5B.

Figure 9 shows a time course of ethoxycoumarin O-deethylase activity in THLE-5B-CMV-1A2 cells.

Figure 10 shows the cytotoxicity of Aflatoxin B_1 in THLE-5B-CMV-1A2 and THLE-5B-CMV-neo lines.

Figure 11 shows the cytotoxicity of Aflatoxin B1 in BEAS-2B-CMV-3A4, BEAS-2B-CMV-2A6, BEAS-2B-CMV-1A2 and BEAS-2B-CMV-neo cell lines.

Figure 12 shows the cytotoxicity of Aflatoxin B_1 or Aflatoxin G_1 in BEAS-2B-pXT1 and BEAS-2B-1A2 lines.

Figure 13 shows PhIP cytotoxicity in THLE-5B-CMV-1A2 and THLE-5B-CMV-1A2 lines.

Figure 14 shows diethylnitrosamine cytotoxicity in BEAS-2B-CMV-2E1 and BEAS-2B-CMV-neo cell lines.

Figure 15 shows dimethylnitrosamine cytotoxicity in BEAS-2B-CMV-2E1 and BEAS-2B-CMV-neo cell lines.

DESCRIPTION OF THE INVENTION

The above and various other objects and advantages of the present invention are achieved by (a) constructing recombinant vectors containing cDNA sequences encoding cytochrome P450 proteins so that mammalian, especially human,

cells when infected or transfected with said recombinant vectors efficiently express the P450 proteins; and (b) providing functionally intact cell lines containing cytochrome proteins without requiring the extraneous addition of NADPH cytochrome P450 reductase for enzymatic activity.

Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. Although any methods and materials similar or equivalent to those described can be used in the practice or testing of the present invention, the preferred methods and materials are now described. All publications and patent documents referenced in this application are incorporated herein by reference.

I. Immortalized cell lines

The cytochrome-P450-expressing, nontumorigenic, stable, immortalized cell lines of the invention are derived from lung and liver immortalized cells. Immortalized cells are preferred over primary cells for use as a testing system because of greater reproducibility of results and less onerous preparation for use (once an immortalized cell line has been established). Immortalized cell lines derived from lung and liver tissues serve as model toxicity systems for the respective tissues from which they were derived. Non-tumorigenic immortalized cells are particularly advantageous because of their greater similarity to normal tissue cells, and because they can be used for determining carcinogenic potential of test substances. The term non-tumorigenic is used to describe cells that do not form tumors when subcutaneously injected into a test animal, such as a mouse. The P450-expressing immortalized cell lines of the present invention are stable in the sense that no detectable reduction of P450 expression occurs after introduction of an exogenous P450 gene for at least 50 passages of the cells.

An immortalized cell line is prepared from cells obtained from a specific tissue of a single human donor. A

homolog of that cell line is a second cell line prepared by the same method from the same tissue, but from a different donor. For example, the cell lines THLE-2, THLE-3 and THLE-5 are homologs (see Example 1). Different clonal isolates of a cell line are referred to as derivative cell lines. For example, cell lines THLE-5B-c15.3 and THLE-5B-c15.4 are derivatives of the THLE-5B cell line.

Immortalized cells preferably retain expression of phase II enzymes, such epoxide hydrolase, catalase, glutathione peroxidase, superoxide dismutase and glutathione S-transferase. These enzymes are involved in detoxification of xenobiotics, and their presence increases the authenticity of cellular toxicity testing system as a model for human tissues.

Although immortalized cells lines are preferred over primary cells for use as toxicity testing systems for the reasons discussed above, it has been observed that existing immortalized cell lines do not express, or express at only low levels, one or more P450 cytochromes. The P450 enzymes are required for metabolic processing of certain xenobiotics to toxic, mutagenic or carcinogenic forms. Thus, the immortalized cells of the present invention are transfected with one or more exogenous cytochrome P450 gene to supplement the expression products of endogenous genes. The exogenous P450 gene(s) are operably linked to expression vector(s) such that the gene(s) are capable of being expressed to produce functional P450 enzymes. Functional P450 enzymes are capable of metabolizing one or more of the substrates in Table 1.

II. Cytochrome P450 Genes and Vectors

Genomic or cDNA clones encoding cytochrome P450 genes may be isolated using hybridization probes designed on the basis of the nucleotide or amino acid sequences for the desired gene. The probes can be constructed by chemical synthesis or by polymerase chain reactions using primers based upon sequence data to amplify DNA fragments from pools or libraries. (US Patents 4,683,195 and 4,683,202.) Nucleotide substitutions, deletions, additions, and the like also may be

incorporated into the cytochrome P450 DNA fragment to be cloned, so long as the biological function of the expression product is not substantially disrupted. (Maniatis, et al, Molecular Cloning: A Laboratory Manual, 2nd Ed. 1989 and Berger and Kimmel, Methods in Enzymology, Volume 152, Guide to Molecular Cloning Techniques (1987). The clones may be expressed or the P450 gene of interest can be excised or synthesized for use in other systems. The sequences of various cDNA isolates are described for cytochrome P4502C9 (Umbenhauer, et al., Biochem., 26:1094-1099, 1987 and Kimura, et al., Nucl. Acids Res., 15:10053-10054, 1987); P4502E1 (Song, et al., J. Biol. Chem., 261:16689-16697, 1986 and Umeno, et al, Biochem., 27:9006-9013, 1988); and P4503A4 (Beaune, et al., Proc. Natl. Acad. Sci. U.S.A., 83:8064-8068, 1986 and Gonzales, et al., DNA, 7:79-86, 1988). Cytochrome P450 1A2 is described by Jaiswal, et al., Nucl. Acids Res., 14:6773-6774, 1986; 2A3 by Yamano, et al., Biochem., 29:1322-1329, 1990; and 2D6 by Gonzalez, et al., Genomics, 2:174-179, 1988.

The members of the cytochrome P450 family differ from each other in substrate specificity and in the tissue types in which they are characteristically expressed. Table 1 shows the tissues in which the various p450 cytochromes are characteristically expressed and also lists suitable carcinogenic substrates for testing for the expression of a particular P450 cytochrome in a cell line. The various members of the cytochrome P450 family are sometimes referred to by abbreviations. For example, CYP1A1 refers to cytochrome P450 1A1; CYP1A2 refers to cytochrome P450 1A2, and so forth. The term "P450 gene" includes genes that hybridize with known P450 genes under stringent conditions, or whose expression products specifically bind to antibodies against known P450 enzymes.

TABLE 1

Human Cytochrome P450s: Tissue distribution and carcinogen activation

Family	Member	Tissues	Carcinogens*
1A	1A1 1A2	In, Li, Lu, Pla, Skin Li	B(a)P AAF, AF, AFB_1, IQ, MeIQ, NNK
2A	2A6	Li, NE	AFB_1, DEN, DMN, NNK
2B	2B6 2B7	Li Li, Lu	AFB_1, NNK AFB_1
2D	2D6	Li	NNK
2E	2E1	Li, Lu, In	DEN, DMN, NNK
3A	3A3, 3A4	Li, Lu, In	AFB_1, AFG_1, B(a)P7,8-diol

B(a)P, Benzo(a)pyrene; **AAF**, acetylaminofluorene; **AF**, aminofluorene; **IQ**, 2-amino-3-methylimidazo(4,5-f)quinoline; **MeIQ**, 2-amino-3,4-dimethylimidazo(4,5-f)quinoline; **DEN**, N-nitrosodiethylamine; **DMN**, N-nitrosodimethylamine; **AFB_1**, aflatoxin B_1; **AFG_1**, aflatoxin G1; **B(a)P7,8-diol**; **NNK**,4-(methylnitro-samino)-1-3-pyridyl)1-butanone

Li, liver; **Lu**, Lung; **In**, intestine; **Pla**, placenta; **NE**, nasal epithelium.

*Carcinogens selected for the evaluation of the established cell lines.

III. Expression Systems

The cytochrome P450 genes can be transferred into the cell lines by transfection of plasmid DNA or by retroviral infection. The viral vector is preferably replication defective so that stable cell lines expressing P450 genes are obtained. Transfection of cells can occur through those methods commonly used, such as calcium or strontium phosphate treatment, microinjection, electroporation, or lipofection. For example, the cells may be infected with a molony-LTR driven promoter or a vaccinia virus or lipofected with an adenovirus-promoter, HIV-promoter or CMV-promoter construct. The transfected DNA plasmid can contain a selectable marker gene or be co-transfected with a plasmid containing a selectable marker, and in some cases, the retroviral vector contains a selectable marker gene. Where one or more selectable marker is transferred into the cells along with the P450 gene, the cell

populations containing the P450 gene can be identified and enriched by selecting for the marker or markers. Markers typically are antibiotic resistant to such antibiotics as tetracycline, hygromycin, neomycin, and the like.

IV. Utility of Cell Lines

The immortalized, nontumorigenic, stable, P450-expressing cell lines of the present invention are useful in the following respects.

(1) Identification of potential chemopreventive drugs. These cells are useful for screening chemicals suitable for the treatment of cancer and related diseases, by growing them in vitro in medium containing the chemical to be tested and then, after a suitable period of exposure, determining whether and to what extent genotoxicity, DNA adduct formation, mutagenicity, cell transformation and/or cytotoxicity has occurred following exposure to a carcinogen, e.g., by trypan blue exclusion assay or related assays (Paterson, Methods Enzymol., 58:141, 1979), or by growth assays such as colony formatting efficiency (MacDonald, et al., Exp. Cell. Res., 50:417, 1968), all of which are standard techniques well known in the art. Once a potential anticarcinogenic agent is identified, it and the cells can be used in further studies, such as drug design.

(2) Studies of the control of squamous differentiation, and identification of chemical and biological agents which induce squamous differentiation (bronchial cells only). This is accomplished by assays previously described for normal human bronchial epithelial cells (Masui, Proc. Natl. Acad. Sci. U.S.A., 83:2438, 1986). Some cells retain the ability to undergo squamous differentiation in response to serum. Induction of terminal differentiation may be an effective way of controlling the growth of cancer. Chemical and biological substances are screened for their ability to induce differentiation by adding them to the growth medium of these cells and then after a suitable time interval determining whether a complex of changes including cessation of DNA

synthesis and the appearance of squamous morphology has occurred. The cells are also useful for studies for the biological mechanisms of squamous differentiation, and the existence of both serum-resistant and serum-sensitive cell lines enables comparisons and identification of genes involved in the process of differentiation.

(3) Programmed cell death. The cell lines are also used for identifying agents that induce programmed cell death or apoptosis, which may have an important impact on prevention of malignant transformation. Programmed cell death is assayed by DNA fragmentation or cell-surface antigen analysis.

(4) Use of recombinant DNA expression vectors to produce proteins of interest. For example, the gene encoding a protein of therapeutic value may be recombined with controlling DNA segments (i.e. containing a promoter with or without an enhancer sequence), transferred into the cell (e.g., by strontium phosphate transfection) and then the protein produced may be harvested from the culture supernatant or a cellular extract by routine procedures well known in the art.

(5) Studies of metabolism of carcinogens and other xenobiotics. Carcinogens and other xenobiotics may be added to the growth medium of these cells and then the appearance of metabolic products of these compounds may be monitored by techniques such as thin layer chromatography or high performance liquid chromatography and the like.

(6) Studies of DNA mutagenesis. Substances known or suspected to be mutagens, or precursors of mutagens, may be added to the growth medium of the cells and then mutations may be assayed, e.g., by detection of the appearance of drug resistant mutant cell colonies (Thompson, Methods Enzymol., 58:308, 1979). Similarly, cell-mediated DNA mutagenesis, by co-cultivating the cells with cell types known or suspected to be capable of secreting mutagenic compounds (Hsu, et al., Proc. Natl. Acad. Sci. U.S.A., 75:2003, 1978).

The P450 enzyme can also be linked to a mutagen detection assay such as the Ames Salmonella/microsome system for detecting or testing the mutagenic frequency induced by

environmental pollutants, carcinogens and the like (Ames, et al., Mut. Res., 31:347, 1975). Other standard methods well known in the art such as chromosome aberration and sister chromatic exchange induction in Chinese hamster ovary cells (Galloway, et al., Environ. Mutagen., 7:1, 1985) or mouse lymphoma cell mutagenesis assays (Myhr, et al., Prog. in Mut. Res., 5:555-568, 1985) can, of course, also be used for testing mutagenicity.

(7) Studies of chromosome damaging agents. Substances known or suspected to cause chromosomal damage may be added to the culture medium of these cell lines, and then the extent of chromosomal damage may be measured by techniques such as measurement of the frequency of sister chromatic exchange (Latt, et al., In: Tice, R.R. and Hollaender, A. Sister Chromatic Exchanges, New York: Plenum Press, pp. 11 ff., 1984).

(8) Studies of malignant transformation. Chemical, physical and viral agents, and transferred genes including oncogenes, mutant tumor suppressor genes, and high molecular weight genomic DNA from tumors are introduced into cells and malignant transformation is determined using standard assays such as anchorage independent growth or tumor formation in athymic nude mice.

(9) Screening for potential chemotherapeutic agents. Cells altered by transfer of oncogenes or chemical carcinogens (as in paragraph 7 above) are used to screen for chemotherapeutic agents by tests which examine reversion of the transformed phenotype of cells by reduction of 50bb agar growth or reduced tumor formation in nude mice.

(10) Studies of cellular biochemistry. For example, changes in intracellular pH and calcium levels are correlated with cell growth and action of exogenous agents including, but not limited to, those described in paragraphs 1 through 9 above. To study intracellular pH and calcium levels, cells in suitable culture vessels are exposed to fluorescent indicator dyes and then fluorescence emissions are detected

with a fluorescence spectrophotometer (Grynkiewicz, et al., J. Biol. Chem., 260:3440-3450, 1985).

(11) Studies of cellular responses to growth factors and production of growth factors. The cells may be used to identify and purify growth factors important for growth and differentiation of human bronchial and liver epithelial cells. The cells of the present inventions are particularly useful for such an application since they grow in serum-free media. Therefore, responses to growth factors can be studied in precisely defined growth media and any factors produced by the cells may be identified and purified without the complication of the presence of serum.

(12) Studies of intracellular communication, e.g., by dye scrape loading assays. To determine whether the cells growing in vitro have the ability to communicate via gap junctions; the cultures may be scraped, e.g. with a scalpel in the presence of a fluorescent dye in the growth medium. Cells at the edge of the wound are mechanically disrupted and therefore take up dye; whether intercellular communication has occurred may be ascertained by determining whether cells distant from the wound also contain dye.

(13) Characterization of cell surface antigens. The cells are incubated with an antibody against the cell surface antigen of interest, and then reacted with a second antibody which is conjugated to a fluorescent dye. The cells are then evaluated using a fluorescence activated cell sorter to determine whether they are fluorescent and therefore possess the cell surface antigen.

(14) Hybrid studies for identification of tumor suppressor activity. To determine whether these cell lines contain tumor suppressor genes, they are fused to malignant tumor cells. The presence of tumor suppressor genes is indicated by loss of malignancy, e.g., as detected by loss of ability to form tumors in athymic nude mice, in the hybrid cells. See Stanbridge, et al., Science, 215:252-259, 1982.

(15) Identification of novel genes. Novel genes, including transforming genes in naturally occurring cancers

described in paragraph 8 above, growth factor genes as described in paragraph 11 above, tumor suppressor genes as described in paragraph 14 above, using standard molecular biological techniques (Davis, et al., Methods in Molecular Biology, New York: Elsevier, 1986) and techniques such as cDNA subtraction cloning and the like. These genes or their derivatives can be used in gene therapy.

Of course, kits for screening carcinogenic or antineoplastic agents and for any other usage as described herein, are easily assembled, comprising container(s) containing the cell line(s) of the present invention, media for propagating cells, and reagents and/or apparatus for detecting morphological, physiological and/or genetic responses in the cell lines. Other components routinely found in such kits may also be included together with instructions for performing the test.

Examples

Example 1. Preparation of Immortalized Cells

A. Bronchial Cells

The immortalized human bronchial epithelial cell lines used in producing the cytochrome P450-transfected cells of the present invention are described in US Patent 4,885,238. These cell lines are prepared as follows.

Normal human bronchial epithelial (NHBE) cells were cultured from explants of necropsy tracheobronchial specimens from noncancerous individuals as described by Lechner, et al., J. Tissue Culture Methods, 9:43-48, 1985. The NHBE cells were infected with adenovirus-12 SV40 hybrid virus. In all cases the life-span of these cultures was extended compared to NHBE; most of the cultures underwent a prolonged period of senescence referred to as "crisis." With continued culture, in some cases colonies of cells which had escaped senescence arose; such surviving colonies were subsequently passaged for extended periods of time and showed unlimited growth potential.

Like NHBE cells, but unlike bronchial carcinoma cells, some of the cell lines thus derived retained the capacity to undergo squamous differentiation in response to serum exposure. Injection of these cells into irradiated athymic nude mice did not result in formation of tumors after periods of up to nine months. Furthermore, these cell lines were found to be suitable recipients for transfection of additional genes and useful for testing the cytotoxicity potential of chemical and physical agents, the growth inhibition or promoting capability of biological agents, and squamous differentiating potential of chemical and biological agents.

Development of the BEAS-2B Cell Line

A preferred cell line for use in this invention is BEAS-2B which was prepared as follows. NHBE cells were cultured from explants of autopsy specimens from noncancerous individuals as described by Lechner, et al., J. Tissue Culture Methods, 9:43-48, 1985. The cells were cultured in a serum-free medium, LHC-9, harvested by trypsinization and seeded in 10 ml growth medium into 100 mm culture dishes (Lux, Miles Scientific, Naperville, IL) whose growth surfaces had been coated with a solution of bovine serum albumin, fibronectin and collagen (Lechner, et al., supra.).

Adenovirus 12-SV40 (Ad12SV40) hybrid virus (Schell, et al. Proc. Natl. Acad. Sci. U.S.A. 55:81-88, 1966) was grown in Vero cells as described by Rhim, et al., Proc. Natl. Sci, U.S.A., 78:313-317, 1981. NHBE cells were exposed to the virus at 37°C for four hours at a multiplicity of infection of approximately 100. When the cultures reached confluence, each dish was subcultured into two 75 cm^2 flasks. The cells were allowed to reach confluence again and then were re-fed twice weekly until transformed colonies appeared and the normal cells senesced. Senescence of the normal cells was accelerated by exposing the cultures to 1% FCS in LHC-9 for 28 days (Lechner, et al., Differentiation, 25:229-237, 1984); all subsequent culture of these cells was in serum-free LHC-9 medium.

Individual colonies were subcultured 41 days after the viral infection and cell strains thus derived from this experiment were designated BEAS-2B. Northern blots of BEAS-2B cells have shown that these cells express phase II enzymes epoxide hydrolase, catalase, glutathione peroxidase, superoxide dismutase and glutathione S-transferase.

When supplemented with exogenous cytochrome P450s, BEAS-2B cells represent an authentic model system for analysis of normal lung tissue in vivo. BEAS-2B cells, are derived from human bronchial epithelial cells, which are the likely progenitor cells of all types of lung cancer. Moreover, except for cytochrome P450s, which are expressed at reduced levels or absent, the BEAS-2B cells express many enzymes involved in the activation process of carcinogens and mutagens, such as glutathione S-transferase, epoxide hydrolase, NADPH cytochrome P450 reductase. BEAS-2B cells have been deposited under the terms of the Budapest Treaty at the American Type Culture Collection and assigned the accession number CRL9609.

B. Liver Cell Lines

The preparation and properties of immortalized, nontumorigenic, human liver cell lines (before transfection with exogenous P450 genes) are discussed in some detail in co-pending application, USSN 07/844,873. Pertinent details of the preparation of cell lines are also described below. Properties of the cell lines are summarized.

(1) Preparation

(a) Primary culture of normal adult liver tissue

LCM medium (Lechner, J. F. et al., Cancer Detect. Prev. 14: 239 (1989)) consists of PFMR-4 medium (Biofluids, Rockville, MD) wherein the Ca^{2+} concentration is reduced to 0.4 mM and arginine is replaced with 0.3 mM ornithine, supplemented with insulin (1.45 μM), transferrin (125 nM), cholera toxin (300 pM), epidermal growth factor (825 pM), hydrocortisone (0.2 μM), triiodothyronine (10 nM), retinoic acid (10 nM), phosphoethanolamine (0.5 μM), Ex-Cyte V (312 μM), bovine pituitary extract (7.5 μg protein/ml), and chemically denatured serum.

To make LCM medium conditioned by Hep-G2 cells (HGLCM), Hep-G2 cells (American Type Culture Collection, Rockville, MD) were maintained in DMEM medium supplemented with 10% fetal bovine serum. Near-confluent cultures of such cells were washed twice with LCM and then maintained in LCM for 72 hours. The supernatant medium (HGLCM) was removed, sterilized by filtration through a 0.22 μm membrane and stored under sterile conditions.

Normal liver epithelial cells were obtained by collagenase/dispase perfusion of the left lower lobe of livers from immediate autopsy adult donors with no clinical evidence of cancer (Hsu, I. C. et al., In Vitro Cell Develop. Biol. 21: 154 (1985)). Cultures were inoculated into flasks that had been precoated with collagen I (Vitrogen™, Celtrix Laboratories, Palo Alto, CA) and incubated overnight in Waymouth's medium containing 10% fetal bovine serum. The following day, the cultures were rinsed with phosphate buffered saline (PBS) and the medium was changed to HGLCM.

Within 2 to 4 days of isolation of the normal cells, groups of randomly spaced replicating cells with an epithelial-like morphology were evident. These cultures formed a confluent monolayer after 10-14 days of incubation. These normal cells could be subcultured at a 1:4 split ratio using the same collagenase/dispase solution as was used in establishing the primary culture to remove the cells from the surface of the culture vessel. The average lifespan of these normal liver epithelial cell cultures was 12 population doublings.

(b) Production of the SV40 Tag-expressing retrovirus

A recombinant retrovirus carrying the large T antigen gene of SV40 was constructed by insertion of BglII-HpaI fragment of the SV40 viral DNA (nucleotides 5235-2666) into the BamHI site of the pZipNeoSVX (Jat., P. S. et al., Mol. Cell. Biol. 6: 1204 (1986)) retroviral vector, using BamHI linkers and standard recombinant DNA techniques. The fragment of the SV40 genome employed lacked both the early promoter and the polyadenylation site.

Infectious recombinant virus particles were made by infecting the amphotropic packaging cell line PA317 with the ecotropic recombinant virus established by transfecting the vector obtained above into the ecotropic packaging line Psi2. Transfected cells were isolated by neomycin selection and 10 clones were isolated. The cloned PA317 cells were propagated in DMEM medium supplemented with 10% FBS. The medium was changed to serum-free PC-1 medium (Ventrex Laboratories, Portland, ME) and collected virus was titered by infecting 8×10^4 NIH 3T3 cells in a 60 mm dish with various dilutions of the supernatant medium containing virus in the presence of 8 μg/ml polybrene and counting the colonies after 10 days of selection using 750 μg/ml of neomycin.

(c) infection of primary liver tissue culture cells

A pool of virus from 7 of the 10 clones of the transfected PA317 cells was used to infect the primary liver tissue cultures. 8×10^4 cells of the primary cultures were infected with the recombinant virus for 2 hours in the presence of 8 μg/ml polybrene in PC-1 medium. After the infection, the cultures were washed with HEPES buffered saline (HBS) and incubated in LCM medium. Infection with the recombinant virus caused virtually all of the liver cells in the culture to undergo rapid division. Several cultures have been so established. All of these have been passaged as mass cultures. Initially, the THLE cells underwent approximately 25 population doublings during the first six weeks post-infection, then growth decreased markedly. Cells were cryopreserved at each passage during this early growth period.

The THLE-2 cell line was deposited under the terms of the Budapest Treaty at the American Type Culture Collection, 12301 Parklawn Dr., Rockville, MD, on May 16, 1989 and assigned the accession number CRL 10149. The THLE-3 cell line was deposited under the terms and conditions of the Budapest Treaty at the American Type Culture Collection on January 14, 1993 and was assigned the accession number CRL 11233. The THLE-5 cell line (also sometimes referred to as "THLE-5B") was deposited under the terms and conditions of the

Budapest Treaty at the American Type Culture Collection on April 23, 1992, and was assigned the accession number CRL11113. The THLE-5B cell lines was used in many of the experiments in Examples 2-5.

(2) Properties of THLE cell lines

Transforming DNA. THLE cells contain approximately one copy of the SV40 T antigen gene as determined by Southern blotting.

Immortality. Growth decreased markedly after ~25 PDs during the first 6 weeks after infection. At this time early-passage-cryopreserved THLE cells were used to determine the growth responses to LCM medium supplements. The clonal growth rate could be optimized by omitting lipid (ExCyte V) and cholera toxin supplements, replacing ornithine with arginine, and replacing HepG2-conditioned medium with T2-CM. With this modified growth medium (MLCM), THLE cells have undergone >130 Pds with no evidence of senescence. Their apparent maximal PD time is 24 hour, and their colony-forming efficiency is ~15%.

Expression of Hepatocyte Phenotypic Traits. Cytokeratin 18, but not cytokeratin 19, was uniformly expressed in early-passage THLE cells, whereas at passage 10-12, all cells also expressed cytokeratin 19. α-Fetoprotein or factor VIII expression was not detected at early- or late-cell passages, whereas α_1-antitrypsin and α_2-macroglobulin were present. Albumin was readily detected in the cytoplasm of early-passage THLE cells by immunocytochemistry. Islands of albumin-positive cells were surrounded by clusters of less intensely staining cells, indicating different cell clones or types. Immunoblot analyses showed that late-passage THLE cells can secrete albumin. The albumin secretion by THLE cells was between ~300 pg/ml and 14.5 ng/ml. γGT was weakly positive by cytochemistry in some colonies of THLE cells, as well as in the primary cultures before introduction of SV40 T antigen. In the same test 3T6 cells were negative, whereas HepG2 cells exhibited high enzyme activity.

Karyotype and Tumorigenicity Analysis. Karyotype analysis showed that THLE cells are hypodiploid with most karyotypes being near-diploid. Typical SV40 T antigen effects were also detected in THLE cells at passage 22 - i.e., monosomy of chromosomes 13 and deletions of chromosomes 2 and 8. When the cell lines were tested for tumor formation by s.c. injection of 10^6 cells per athymic nude mouse (20 animals), no tumors were found after 12 months of observation.

Metabolic Studies. The metabolism, cytotoxicity, and DNA adduct formation of three different chemical classes of carcinogens were investigated in THLE cells. AFB_1, B[*a*]P, or DMN caused dose-dependent cytotoxicity of THLE cells, suggesting metabolic activation of these promutagens to genotoxic metabolites. AFB_1, DMN, or B[*a*]P formed 3.5 ± 0.9, 30.4 ± 3.9, and 1.5 ± 0.1 fmol of adduct per μg of DNA, respectively, in THLE cells grown in roller bottles. The major adduct found in cells treated with ^{3}H-labeled B[*a*]P was chromatographically indistinguishable from the major product formed when (±)%7, *t*-8-dihydroxy-*c*-9,10-epoxy-7,8,9,10-tetrahydrobenzo[*a*]pyrene (BPDE) was allowed to react with DNA. ^{32}P-postlabeling analysis revealed the N^1-methyldeoxyguanosine adduct in THLE cells incubated with DMN. The major adduct in AFB_1-exposed THLE cells was 8,9-dihydro-8-(2,6-diamino-4-oxo-3,4-dihydropyrimid-5-yl-formamido)-9-hydroxyaflatoxin B_1 (AFB_1-FAPyr), whereas AFB_1-diol and 8,9-dihydro-8(N^1-guanyl)-9-hydroxyaflatoxin B_1 were minor adducts. Preincubation of cells with Aroclor 1254, an inducer of CYP1A1/1A2 enhanced formation of B[*a*]P-related adducts 3-fold to 4.9 ± 2.7 fmol/μg of DNA, decreased DMN-related adducts to 3.4 ± 0.1 fmol/μg of DNA, and did not affect AFB_1-DNA adduct formation (1.6 ± 0.4 fmol/μg of DNA). Pretreatment with β-naphthoflavone abolished the ability of THLE cells to activate B[*a*]P. Similarly, ethanol treatment of the THLE cells decreased metabolic activation of DMN.

Expression of Phase I and II Enzymes. RNA analyses of CYP1A1 mRNA steady-state levels were consistent with the results from DNA-adduct analyses. CYP1A1 mRNA was undetectable in control cells grown as roller bottle cultures. Aroclor 1254

or B[a]P exposure increased steady-state levels of CYP1A1 mRNA. When cells were treated with both agents, the CYP1A1-inducing effects with both components appeared additive. In contrast, neither DMN nor AFB_1 induced expression of CYP1A1 mRNA in roller bottle cultures of THLE cells. Other CYPs (CYP1A2, CYP2A3, CYP2E1, CYP2D6, and CYP3A4) were not detectable by RNA blot analysis.

THLE cells express the same amount of epoxide hydrolase mRNA but less NADPH CYP reductase mRNA. Detoxifying enzymes such as superoxide dismutase, catalase, and glutathione peroxidase are expressed in THLE cells at mRNA steady-state levels similar to the amounts found in human liver tissue. GST π mRNA were not found in the donor's liver tissue but were expressed by THLE cells. In contrast, GST-α mRNA was only detected in the original human tissue (data not shown).

Conclusions. These results indicate that THLE cell lines exhibit many of the properties associated with the quiescent state of normal adult hepatocytes, other than the expression of a full complement of cytochrome P450 enzymes. Although THLE cells are capable of some metabolism of toxic, carcinogenic or mutagenic substances, this capacity is much less than that of the P450-transfected THLE cells of the present invention. See Example 5.

Example 2. Introduction of P450 enzymes into immortalized cells

A. pXT1 Defective-Retrovirus Infection System

cDNAs for the cytochrome P450 enzymes, 1A2, 2A3, 3D6, 2E1, and 3A4, were introduced by recombinant high titer amphotropic retroviruses into the BEAS-2B cells. These retroviruses were generated by cloning the corresponding cDNAs into a plasmid pXT1 (Boulter, et al., Nucleic Acid, 15:7194, 1987) and transfecting the recombinant plasmids into co-cultured packaging lines with amphotropic (PA317) and ecotropic envelopes (Psi2) using calcium phosphate precipitation

(Bestwick, et al., Proc. Natl. Acad. Sci. U.S.A., 85:5404-5408, 1988) (see Figure 1).

After 10 days, virus was collected from confluent PA317/Psi2 cultures in serum free PC-1 medium (Ventrex Laboratories, Inc., Portland, OR). The titers were determined on NIH3T3 cells and were expressed as neomycin resistant colonies/ml supernatant. The BEAS-2B cells were infected for 2 hours with the P450 viruses or the control virus, pXT1, in PC-1 medium supplemented with 8 μg/ml polybrene (Table 2).

TABLE 2

Generation of high titer amphotropic P450 retroviruses

Retrovirus	Titer	Metabolic Activity
1A2	10^5	AA, HAA, MeiQ, NNK, AFB_1, caffeine
2A3	2×10^5	DEN, DMN, NNK, AFB_1, coumarin
2D6	5×10^4	buforol, debrisoquine, NNK
2E1	10^5	DEN, DMN, NNK, Ethanol
3A4	6×10^4	AFB_1, B(a)P 7,8-diol, nifedipine

AA, aromatic amines; HAA, heterocyclic aromatic amines; NNK, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone; AFB_1, aflatoxin B_1; DEN, diethylnitrosamine; DMN, dimethylnitrosamine; MeiQ, 2-amino-3,8-dimethylimidazo[4,5-f]quinoxaline.

An equal ratio of cells to colony forming units of the virus was employed. Forty-eight hours after infection the BEAS-2B cells were selected for G418 neomycin resistance with 125 μg/ml neomycin for 8 days. Subsequently, the cells were selected for the presence of the introduced genes by Western blot analysis. Exemplified for BEAS-2B-1A2, the population and 3 clones (clone 8 > clone 3 > clone 6) expressed the protein corresponding to the respective P450 retrovirus. In accordance, clone 8 (cl 8) showed the highest sensitivity being up to 150 times more responsive to the cytotoxic effect and up to 250 times to the genotoxic effect of a model compound, AFB_1, than the control.

B. CMV-Plasmid Lipofection System

pCMV-cytochrome-P450 constructs were generated by cloning human cytochrome P450 cDNAs at the 3' side of the promoter of the cytomegalovirus (CMV) immediate-early gene region. See Figure 2. cDNA fragments were inserted into the BamHI site (after modifications of the sticky ends) of the pCMV neo plasmid (kindly provided by Bert Vogelstein, Johns Hopkins University) to generate the pCMV-cytochrome P450 constructs. Construction of pCMV is described by Baker et al., Science (1990) 249: 912-915. pA is the polyadenylation sequence of the rabbit β-globin gene. Neo is the selectable neomycin gene, conferring G418 selection resistance. Amp^R is ampicillin resistance gene.

pCMV-cytochrome-P450 constructs (and unmodified pCMV vector as a control) were introduced into liver and bronchial cell lines by lipofection. Briefly, 1.10^6 cells were lipofected with 10 μg DNA in 5 ml of Opti-MEM medium (GIBCO-BRL) containing 50 μl of Lipofectin (GIBCO-BRL). After 3 hours the cells were washed and fresh medium containing 10% chemically denatured fetal bovine serum (Upstate Biotechnology, Inc., New York) was added. After 48 hours the transfected THLE-5B or BEAS-2B cells were selected for G418 resistance with 50 μg/ml G418 for two weeks.

Example 3. Immunoblot analysis of introduced P450 genes

After introduction of the P450 genes by replication-defective retroviral infection or lipofection, as described in Example 2, cell lines were tested for expression of P450 genes by Western blotting. Samples of total protein extract (approximately 2.10^4 cells) and standard human cytochrome P450 microsomal fractions (10 μg) (Gentest Corp., Woburn, MA) (as positive controls) were subjected to SDS-PAGE (15% polyacrylamide gels) and transferred to nitrocellulose membranes using a semi-dry electroblotter (Ancos, Denmark). The filters were incubated with polyclonal antibodies against the cytochrome P450 cytochrome under test (diluted 1:50) and developed using an ImmunoPure ABC alkaline phosphatase rabbit

IgG staining kit (Pierce, Socochim SA, Switzerland). Figures 3-7 show expression of P450 cytochromes 1A2, 2A6, 3A4, 2E1 and 2D6 in respective BEAS-2B cells that have been transfected with the respective gene linked to the pCMV vector. Also shown are standard microsomal fractions (M) as positive controls and BEAS-2B cells transfected with unmodified pCMV (B-CMV-neo) as negative controls. These results indicate that transfected exogenous P450 cytochrome genes are expressed in BEAS-2B cells. Similar results were obtained after transfected of THLE-5B cells. Figure 8 shows that expression of cytochrome P450-1A2 was obtained in THLE-5B-CMV 1A2 cell lines. No expression was observed in control THLE-5B-CMV cells (which lack an exogenous P450 gene).

Example 4. Metabolism of cytochrome P450 substrates in cells transfected with exogenous P450 genes

Cells containing exogenous P450 genes were tested for their ability to metabolize P450 substrates thereby demonstrating the functionality of P450 enzymes resulting from expression of the exogenous genes. In one experiment, ethoxycoumarin was used as a substrate to determine functionality of cytochrome P450 1A2 in BEAS-2B and THLE-5B cells. Cultures were plated at 0.25 to 0.5 10^6 cells/60-mm petri dish. On the next day, the medium was replaced with 2 ml of assay buffer (0.2 M sucrose, 0.05 M Tris, pH 8.5, 0.01 M $MgCl_2$) containing 250 μM 7-ethoxycoumarin substrate. After incubation at 37°C for the desired length of time, 1.0 ml of the supernatant was acidified by the addition of 100 μl of 20% TCA. After centrifugation, the supernatant was mixed with 2.0 ml of 1.6 M Glycine-NaOH buffer pH 10.3 and the fluorescence read with excitation at 390 nm and emission 440 nm. Quantitation can be achieved by comparison to the fluorescence of known quantity of umbelliferone. Table 3 shows ECD activity for AHH-1A2/Hyg (lymphoblast cell line containing CYP1A2, described by Crespi, supra), BEAS-2B-1A2 c18 (BEAS-2B cell line containing CYP1A2 linked to the pXT1 expression system), BEAS-2B-CMV-1A2 c12 (BEAS-2B cell line containing

CYP1A2 linked to the pCMV expression system) and THLE-5B-CMV-1A2 c15.3 and THLE-5B-CMV-1A2 c15.4 (different clones of THLE-5B cells containing CYP1A2 cytochrome linked to the pCMV vector. Figure 4 shows a time course for ECD activity of CYP1A2 for the THLE-5B-CMV-1A2 c15.3 and THLE-5B-CMV-1A2 c15.4 cell lines.

TABLE 3

Ethoxycoumarin O-deethylase activity in CYP1A2 expressing cells

	ECD activity (pmol/10^6 cells/min)
AHH-1A2/Hyg	1.25
BEAS-2B-pXT1 c14	und.
BEAS-2B-1A2 c18	0.07
BEAS-2B-CMV-neo c12	und.
BEAS-2B-CMV-1A2 c12	0.21
THLE-5B-CMV-neo c15.16	und.
THLE-5B-CMV-1A2 c15.3	4.3
THLE-5B-CMV-1A2 c15.4	2.0

und., undetectable

In a similar experiment, it was also shown that THLE-5B-CMV-1A2 c15.3 cells are capable of metabolizing ethoxyresofurin substrate about 100-fold more rapidly than THLE-5B-CNV-neo c15.16 control cells. See Table 4. This confirms that the CYP1A2 enzyme resulting from expression of the exogenous gene is also functional for ethoxyresofurin metabolism.

TABLE 4

Ethoxyresorufin-O-deethylase (EROD) activity in CYP1A2-expressing THLE cells

Cell lines	EROD activity in whole cells*
T5-CMV-neo c15.16	0.11 ± 0.04
T5-CMV-1A2 c15.3	9.90 ± 0.40

*pmol/10^6 cells/min

In a further experiment, it was shown that BEAS-2B cells containing exogenous cytochrome P450 2A6 are able to metabolize coumarin at a high rate compared with control cells, indicating functionality of the 2A6 expression product. See Table 5.

TABLE 5

Coumarin-7-hydroxylase (CH) activity in CYP2A6-expressing BEAS-2B

Cell lines	CH-activity in whole cells*
B-CMV-neo c12	und.
B-CMV-2A6 c11	45.7 ± 3.3
B-CMV-2A6 c13	2.0 ± 0.7
B-CMV-2A6 c15	37.0 ± 2.7

*pmol/10^6 cells/min.; und., undetectable

Example 5. Cytotoxicity and Genotoxicity Analysis of P450-expressing Cell Lines

A. AFB_1 Cytotoxicity Analysis

Cultures were exposed to the indicated concentrations of Aflatoxin B_1 (AFB_1). Each culture contained about 1 x 10^5 cells per 60 mm dish. After 28 hours, the cells were washed and fresh medium was added. After 5 days the cell number was determined. Cytotoxicity is expressed as survival relative to the corresponding untreated cells. Values are expressed as the mean ± SD of two independent experiments.

Table 6 and Figure 10 compare the relative survival of different cell types after treatment with various dosages of AFB_1. AHH-1A2/Hyg is the P450-expressing lymphoblast cell line of Crespi, supra, and AHH-1TK+/- is a control lacking an exogenous P450 gene; BEAS-2B-1A2 c18 and BEAS-2B-CMV-1A2 c12 are BEAS-2B cell lines containing an exogenous P450 gene under the respective control of pXT1 and pCMV expression systems; BEAS-2B-pXT1 c14 and BEAS-2B-CMV-neo c12 are control BEAS-2B cell lines containing unmodified pXT1 and pCMV expression

vectors; THLE-5B-CMV-1A2 c15.3 is a THLE-5B line containing CYP1A2 on a pCMV vector, and THLE-5B-CMV-neo c15.16 is a control THLE-5B cell line containing an unmodified pCMV vector. The survival of THLE-5B strains was also determined by a 96-well microtiter assay. In this assay 1 x 10^4 cell/well were treated with AFB_1 for 28 hours. Four to five days, later the cells were stained with crystal violet. After dye extraction, the plates were read at 630 nm.

TABLE 6

AFB_1 cytotoxicity and ECD activity in 1A2-expressing cells

	Relative Survival									
	AHH-1 TK+/- 1A2/Hyg		B-pXT1 cl4[1]	B-1A2 cl8	B-CMV-neo cl2	B-CMV 1A2 cl2	T5;CMV-neo cl5.16[1]		T5-CMV-1A2 cl5.3	
	Relative cell number		Relative cell number				Relative cell number			
AFB_1 (ng/ml)							0.6cm*	86 wells**	0.6 cm	96 wells**
0.05	nd	nd	nd	nd	nd	nd	1.05	1.03	0.96	1.04
0.10	nd	nd	nd	nd	nd	nd	0.91	1.07	0.61	1.02
0.25	nd	nd	nd	nd	nd	nd	0.91	1.10	0.26	0.77
0.5	nd	nd	nd	nd	nd	nd	0.82	1.07	0.07	0.39
1.0	nd	nd	nd	nd	nd	nd	1.00	1.20	0.03	0.15
3.0	nd	0.76	nd	nd	nd	nd	nd	nd	nd	nd
5.0	nd	nd	nd	nd	0.98	0.52	1.08	nd	nd	nd
10.0	nd	0.69	1.04	1.01	1.19	0.37	1.04	0.89	nd	nd
50.0	nd	nd	nd	nd	1.03	0.19	0.83	nd	nd	nd
60.0	1.05	0.26	nd	nd	nd	nd	nd	nd	nd	nd
100.0	nd	nd	0.87	0.27	0.87	0.16	0.75	0.70	nd	nd
300.0	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
1000	0.89	nd	0.76	0.003	0.45	0.04	0.06	nd	nd	nd
10000	0.52	nd	0.13	0	nd	nd	nd	nd	nd	nd

[1]As used in this heading, "B-" refers to "BEAS-2B-"

[2]As used in this heading, "T5-" refers to "THLE-5B"

0.6cm*: 1.10^5cells/0.6cm were treated with AFB_1 during 28 hrs. 5 days later the cells were counted.
96 wells: 1.10^4 cells/well were treated with AFB_1 during 28 hrs. 4 to 5 days later the cells were stained with violet cristal. After elution, the plates were read at 630 nm.

Table 6 shows that all of the cell lines containing an exogenous p450 gene exhibited lower survival than the corresponding controls. The table also indicates that BEAS-2B cells expressing P450 from pCMV are more sensitive than BEAS-2B cells expressing P450 from pXT1, suggesting that operable linkage of a P450 gene to the cytomegalovirus promoter in pCMV supports greater expression of P450. Of the various cell lines tested, the THLE-5B cells containing P450 linked to the pCMV expression system showed the greatest sensitivity to AFB_1.

TABLE 7

AFB_1 cytotoxicity in CYP1A2 expressing cells

	CD50 (AFB_1 ng/ml)
AHH-TK+/-	10000
AHH-1A2/Hyg	25.0
BEAS-2B-pXT1 c14	4500
BEAS-2B-1A2 c18	50.0
BEAS-2B-CMV-neo c12	900
BEAS-2B-CMV-1A2 c12	5.5
THLE-5B-CMV-neo c15.16	200
THLE-5B-CMV-1A2 c15.3	0.15

Table 7 shows the CD50 values derived from the data in Table 6. The CD50 is the dose of carcinogen needed to obtain 50% survival. Similar conclusions can be drawn from this table to those discussed supra for Table 6.

BEAS-2B cultures containing other exogenous cytochrome P450 genes have also been tested for aflatoxin B1 cytotoxicity together with appropriate control cells. Figure 11 shows that BEAS-2B-CMV-3A4 c17 cells (BEAS-2B cells containing an exogenous cytochrome P450 3A4 gene linked to the pCMV vector) and BEAS-2B-CMV-2A6 c15 (BEAS-2B cells containing an exogenous cytochrome P450 2A6 gene linked to the pCMV vector) exhibit greater cytotoxicity than BEAS-2B-CMV-neo c12 cells (BEAS-2B cells containing the pCMV vector but lacking an exogenous P450 gene).

B. AFB_1 Genotoxicity Analysis

Table 8 gives the DNA-adduct formation with AFB_1 for the BEAS-2B-1A2 c18 cell line compared with a BEAS-2B-pXT1 c14 control. These are the same cell lines as are described in Table 6. The formation was elevated by a factor of 1000 in clone 8.

TABLE 8

Binding of $[^3H]AFB_1$ to cellular DNA

Carcinogen exposure (μg/ml)	Adduct formation (pmol/mg DNA)	
	BEAS-2B- pXT1 cl 4	BEAS-2B-1A2 cl 8
-	und.	und.
0.1	und.	0.39
1.0	0.04	9.00

Approximately 1.10^7 cells were exposed to 0.1 or 1.0 μg/ml $[^3H]AFB_1$ (0.2 Ci/mmol) under the conditions of the cytotoxicity assay. Cellular DNA was isolated and binding was measured by liquid scintillation counting (und.: undetectable).

C. AFG_1 Cytotoxicity Analysis

Figure 12 shows the analysis of cytotoxicity for Aflatoxin G_1 in comparison with Aflatoxin B_1 on the BEAS-2B--pXT1 and BEAS-2B-1A2 clones. The cells were exposed to various concentrations of the mutagens for 28 hours. Each culture contained 250 cells per 60 mm dish. After 7-10 days, cytotoxicity was determined by measuring the colony number of each plate. The colony number of the mutagen-treated cultures was divided by the colony number of the untreated cultures to yield relative survival. Each time point reflects at least 3 independent experiments. Figure 12 shows that BEAS-2B-1A2 are more sensitive to both Aflatoxin B1 and Aflatoxin G1 than control cells lacking the exogenous cytochrome P450-1A2 gene.

D. PhIP Cytotoxicity Analysis

THLE-5B cells expressing cytochrome CYP1A2 from the pCMV vector were tested for PhIP cytotoxicity using the method described in Example 5.A. Cultures of cells were exposed to the indicated concentrations of PhIP. Figure 13 shows that the THLE-5B-CMV-1A2 cl 5.3 cell line is far more sensitive to PhIP

than the control strain THLE-5B-CMV-neo c15.18 (which contains the unmodified pCMV vector.)

E. Diethylnitrosame and Dimethylnitrosamine Cytotoxicity analysis

BEAS-2B cells expressing cytochrome P450 2E1 from the pCMV vector were tested for cytotoxicity to diethylnitrosamine and dimethylnitrosamine. Figures 14 and 15 show that these cells are more sensitive to diethylnitrosamine and dimethylnitrosamine than control cell lines containing unmodified pCMV vector.

WHAT IS CLAIMED IS:

1. A non-tumorigenic, stable, human bronchial epithelial cell line containing an exogenous cytochrome P450 gene, capable of being expressed in said cell line.

2. The cell line of claim 1 wherein said exogenous cytochrome P450 gene is 1A1, 1A2, 2A6, 3A3, 3A4, 2B6, 2B7, 2C9, 2D6, and/or 2E1.

3. The cell line of claim 2, which is selected from a group consisting of cell lines BEAS-2B-1A1, BEAS-2B-1A2, BEAS-2B-2A6, BEAS-2B-3A3, BEAS-2B-3A4, BEAS-2B-2B6, BEAS-2B-2B7, BEAS-2B-2C9, BEAS-2B-2D6, BEAS-2B-2E1, and homologs and derivatives of said cell lines.

4. The cell line of claim 3, wherein said exogenous cytochrome p450 gene is operably linked to a cytomegalovirus promoter.

5. The cell line of claim 3, which is BEAS-2B-1A2, or a homolog or derivative thereof.

6. The cell line of claim 1, produced by inserting said exogenous cytochrome P450 gene into a BEAS-2B cell line, deposited as ATCC CRL 9609, or a homolog or derivative thereof.

7. The cell line of claim 6, produced by inserting said exogenous cytochrome P450 gene into said BEAS-2B cell line.

8. A method for identifying or testing the mutagenicity, cytotoxicity or carcinogenicity of an agent comprising the steps of:

a) reacting, culturing, or contacting the cell line of claim 1 with an agent suspected of being a mutagen, cytotoxin, or carcinogen; and

b) determining or monitoring those effects on said cell line which are indicative of mutagenicity, cytotoxicity, or carcinogenicity.

9. The method of claim 8 wherein said cell line is BEAS-2B-1A2.

10. A method for identifying or testing the chemopreventive activity of an agent comprising the steps of:

a) reacting, culturing, or contacting the cell line of claim 1 with an agent suspected of being a chemopreventive in the presence of a carcinogen; and

b) determining or monitoring those effects on said cell line which are indicative of chemopreventive activity.

11. The method of claim 10 wherein said agent is reacted, cultured, or contacted with said cell line prior to the addition of said carcinogen.

12. A method for determining the metabolites activated by a carcinogen or xenobiotic comprising the steps of:

a) reacting, culturing, or contacting the cell line of claim 1 with the suspected carcinogen or xenobiotic; and

b) identifying the metabolites and/or their effects.

13. A diagnostic kit comprising the cell line of claim 1, media for propagating said cell line and reagents for diagnosing a response of said cell line to a carcinogenic, mutagenic or toxic agent.

14. A non-tumorigenic, stable, human adult liver epithelial cell line containing an exogenous cytochrome P450 gene capable of being expressed in said cell line.

15. The cell line of claim 14, wherein said exogenous cytochrome P450 gene is 1A1, 1A2, 2A6, 3A3, 3A4, 2B6, 2B7, 2C9, 2D6, and/or 2E1.

16. The cell line of claim 15 which is selected from a group consisting of cell lines THLE-5B-1A1, THLE-5B-1A2, THLE-5B-2A6, THLE-5B-3A3, THLE-5B-3A4, THLE-5B-2B6, THLE-5B-2B7, THLE-5B-2C9, THLE-5B-2D6, THLE-5B-2E1, and homologs and derivatives of said cell lines.

17. The cell line of claim 16, wherein said exogenous cytochrome P450 gene is operably linked to a cytomegalovirus promoter.

18. The cell line of claim 15, wherein said cell line is THLE-5B-1A2, or a homolog or derivative thereof.

19. The cell line of claim 14, produced by inserting said exogenous cytochrome P450 gene into a THLE-5B cell line, deposited as ATCC CRL 11113, or a homolog or derivative thereof.

20. The cell line of claim 19, produced by inserting said exogenous cytochrome P450 gene into said THLE-5B cell line.

21. A method for identifying or testing the mutagenicity, cytotoxicity or carcinogenicity of an agent comprising the steps of:

a) reacting, culturing, or contacting the cell line of claim 14 with an agent suspected of being a mutagen, cytotoxin, or carcinogen; and

b) determining or monitoring those effects on said cell line which are indicative of mutagenicity, cytotoxicity, or carcinogenicity.

22. The method of claim 21 wherein the cell line is BEAS-2B-1A2.

23. The method for identifying or testing the chemopreventive activity of an agent comprising the steps of:

a) reacting, culturing, or contacting the cell line of claim 14 with an agent suspected of being a chemopreventive in the presence of a carcinogen; and

b) determining or monitoring those effects on said cell line which are indicative of chemopreventive activity.

24. The method of claim 23 wherein said agent is reacted, cultured, or contacted with said cell line prior to the addition of said carcinogen.

25. A method for determining the metabolites activated by a carcinogen or xenobiotic comprising the steps of:

a) reacting, culturing, or contacting the cell line of claim 14 with the suspected carcinogen or xenobiotic; and

b) identifying the metabolites and/or their effects.

26. A diagnostic kit comprising the cell line of claim 14, media for propagating said cell line and reagents for diagnosing a response of said cell line to a carcinogenic, mutagenic or toxic agent.

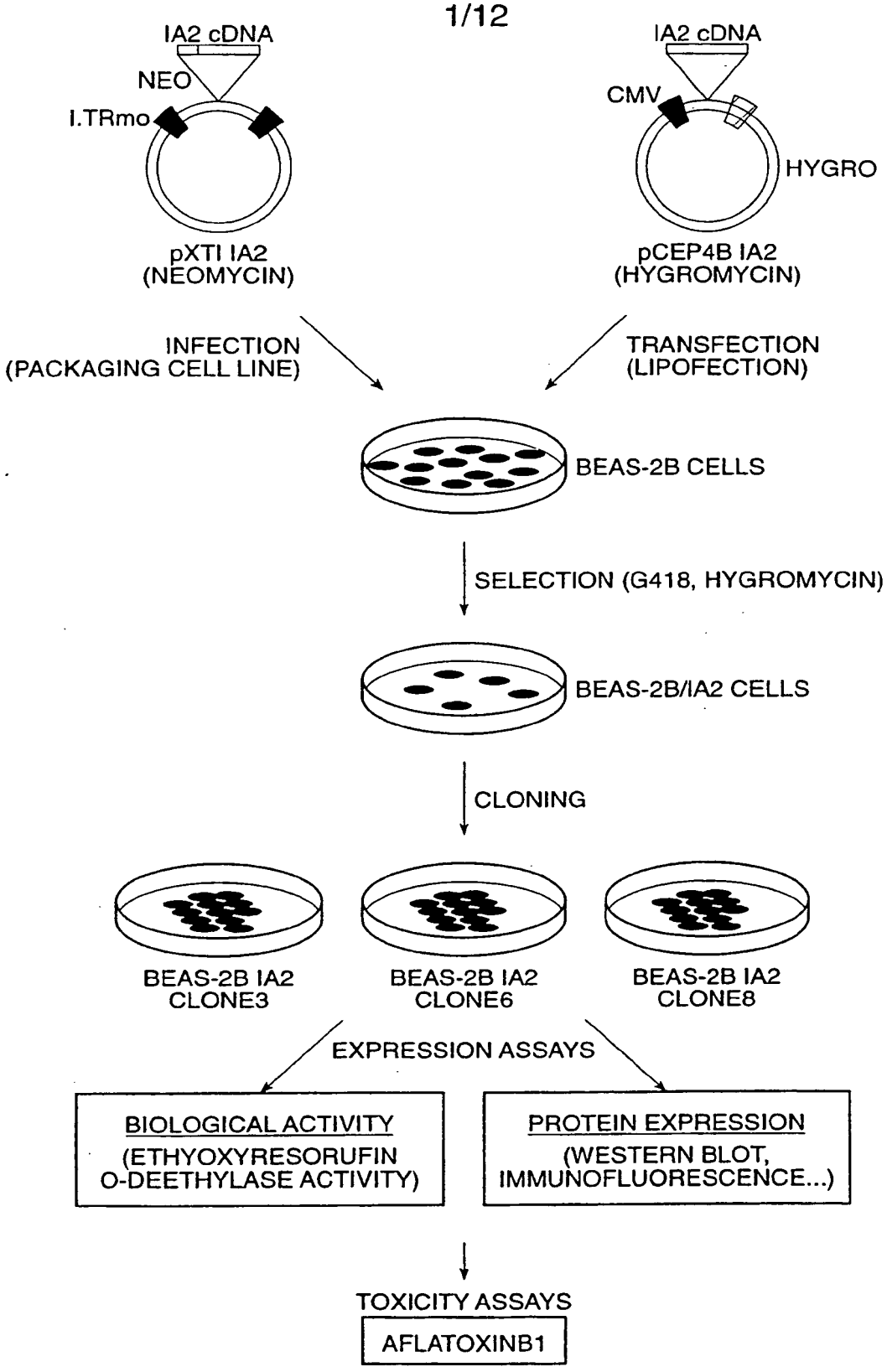
IA2 cDNA
NEO
I.TRmo
pXTI IA2
(NEOMYCIN)
IA2 cDNA
CMV
HYGRO
pCEP4B IA2
(HYGROMYCIN)
INFECTION
(PACKAGING CELL LINE)
TRANSFECTION
(LIPOFECTION)
BEAS-2B CELLS
SELECTION (G418, HYGROMYCIN)
BEAS-2B/IA2 CELLS
CLONING
BEAS-2B IA2
CLONE3
BEAS-2B IA2
CLONE6
BEAS-2B IA2
CLONE8
EXPRESSION ASSAYS
BIOLOGICAL ACTIVITY
(ETHYOXYRESORUFIN
O-DEETHYLASE ACTIVITY)
PROTEIN EXPRESSION
(WESTERN BLOT,
IMMUNOFLUORESCENCE...)
TOXICITY ASSAYS
AFLATOXINB1

FIG. 1

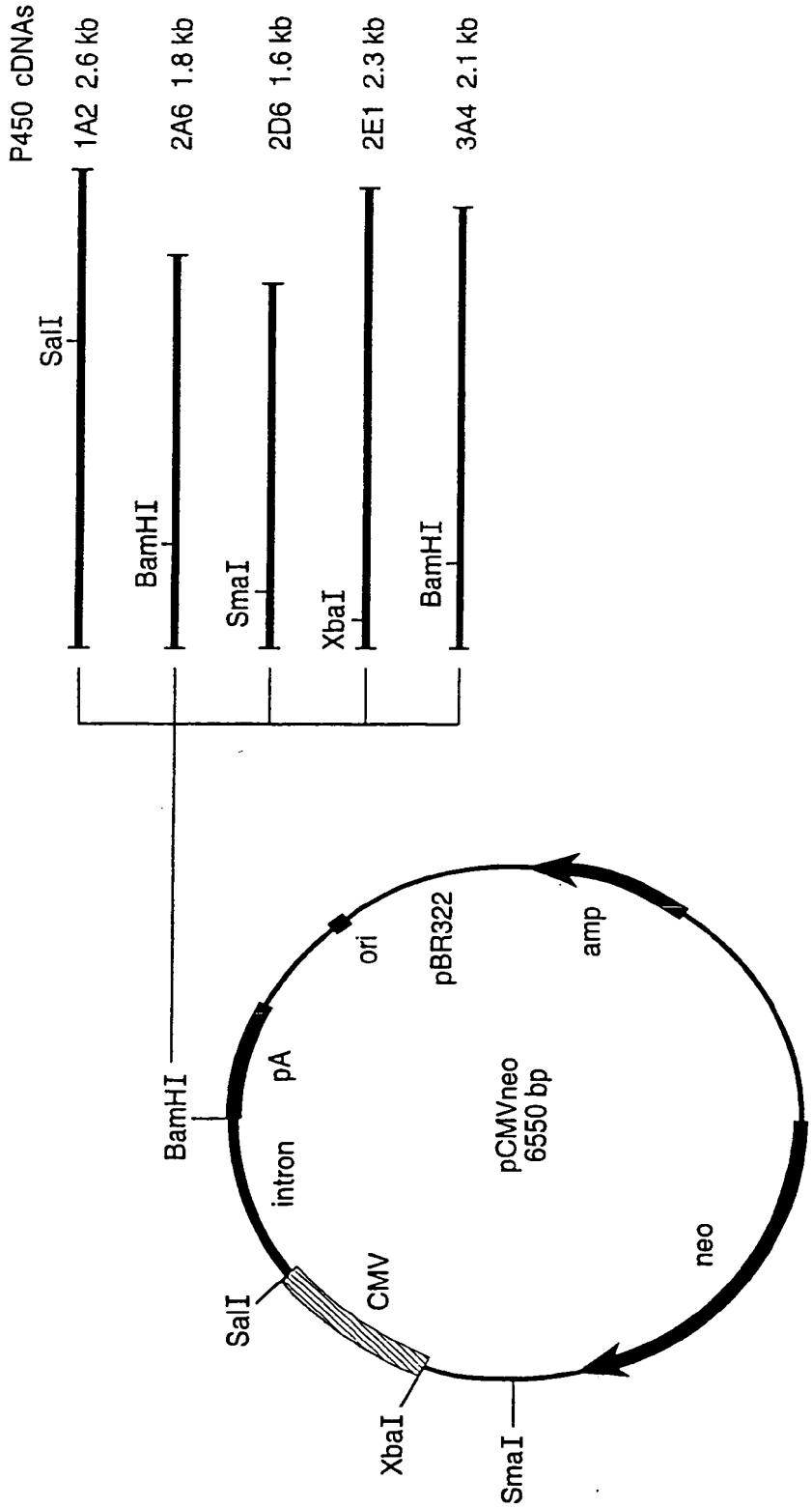
P450 cDNAs
1A2 2.6 kb
2A6 1.8 kb
2D6 1.6 kb
2E1 2.3 kb
3A4 2.1 kb
SalI
BamHI
SmaI
XbaI
BamHI
BamHI
intron
pA
ori
pBR322
amp
pCMVneo
6550 bp
neo
CMV
SalI
XbaI
SmaI

FIG. 2

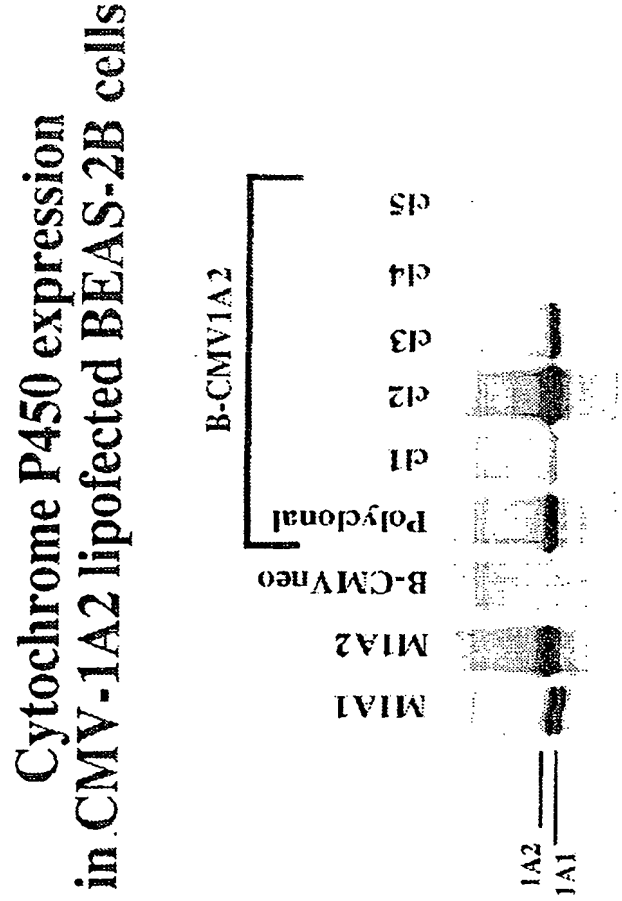
Cytochrome P450 expression
in CMV-1A2 lipofected BEAS-2B cells
B-CMV1A2
cl5
cl4
cl3
cl2
cl1
Polyclonal
B-CMVneo
M1A2
M1A1
1A2
1A1

FIG. 3

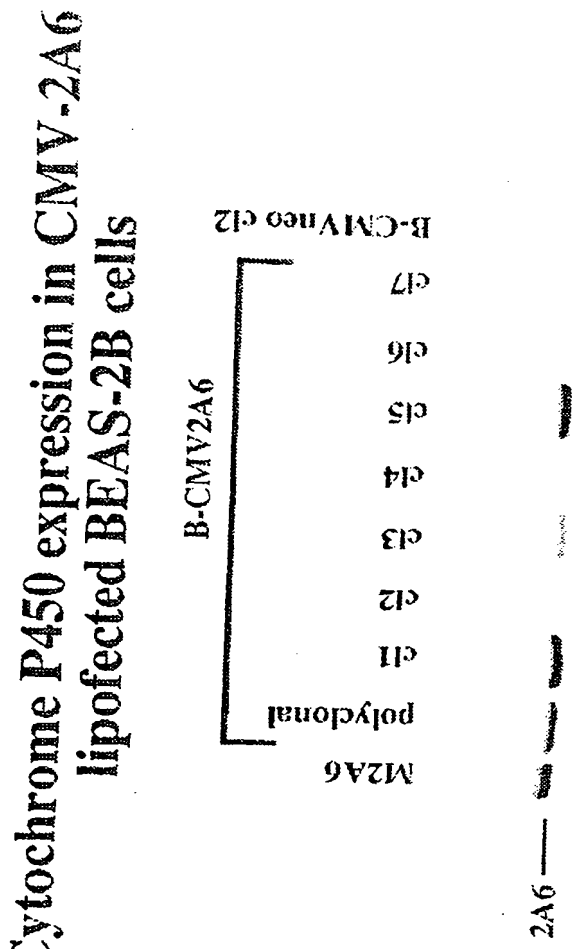
Cytochrome P450 expression in CMV-2A6 lipofected BEAS-2B cells
2A6
M2A6
polyclonal
cl1
cl2
cl3
cl4
cl5
cl6
cl7
B-CMVneo cl2
B-CMV2A6

FIG. 4

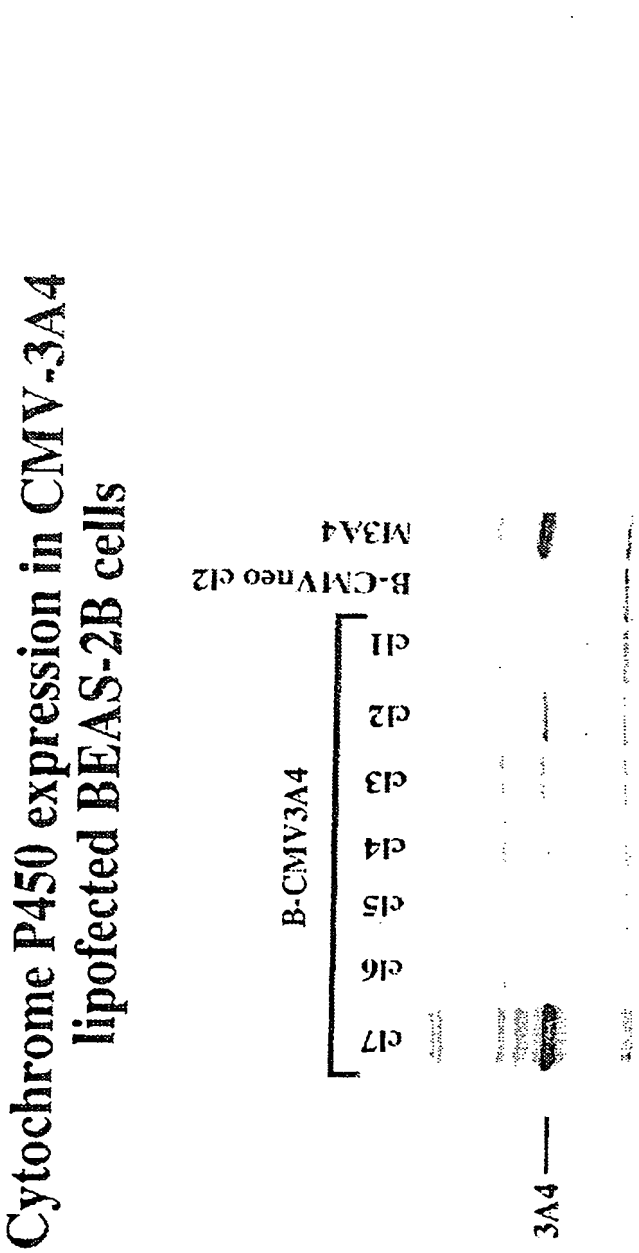
Cytochrome P450 expression in CMV-3A4
lipofected BEAS-2B cells
M3A4
B-CMVneo cl2
B-CMV3A4
cl1
cl2
cl3
cl4
cl5
cl6
cl7
3A4

FIG. 5

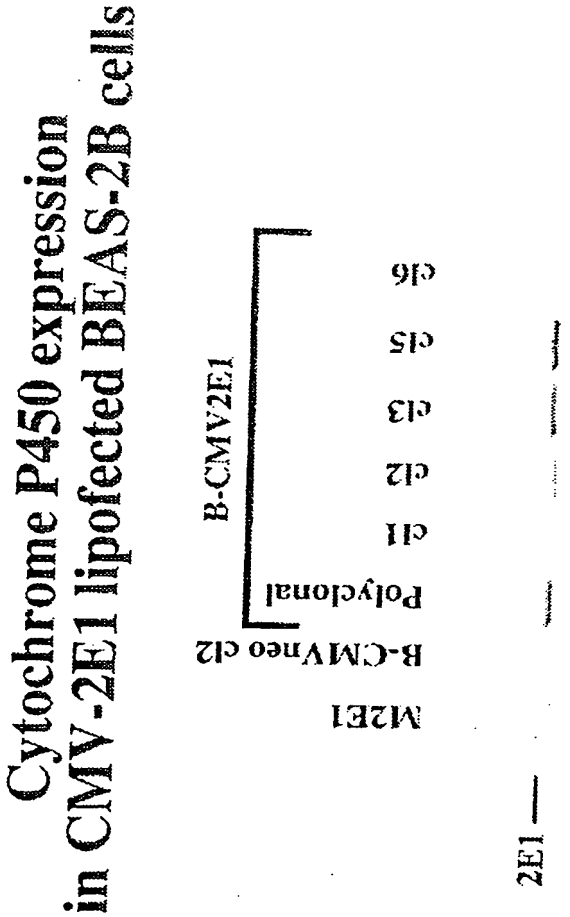
Cytochrome P450 expression
in CMV-2E1 lipofected BEAS-2B cells
2E1
M2E1
B-CMVneo cl2
B-CMV2E1
Polyclonal
cl1
cl2
cl3
cl5
cl6

FIG. 6

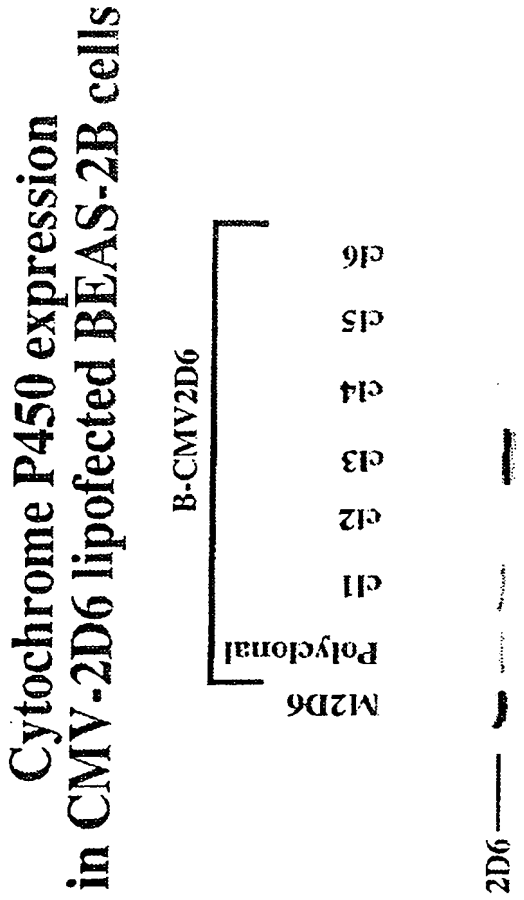
Cytochrome P450 expression
in CMV-2D6 lipofected BEAS-2B cells
B-CMV2D6
M2D6
Polyclonal
cl1
cl2
cl3
cl4
cl5
cl6
2D6

FIG. 7

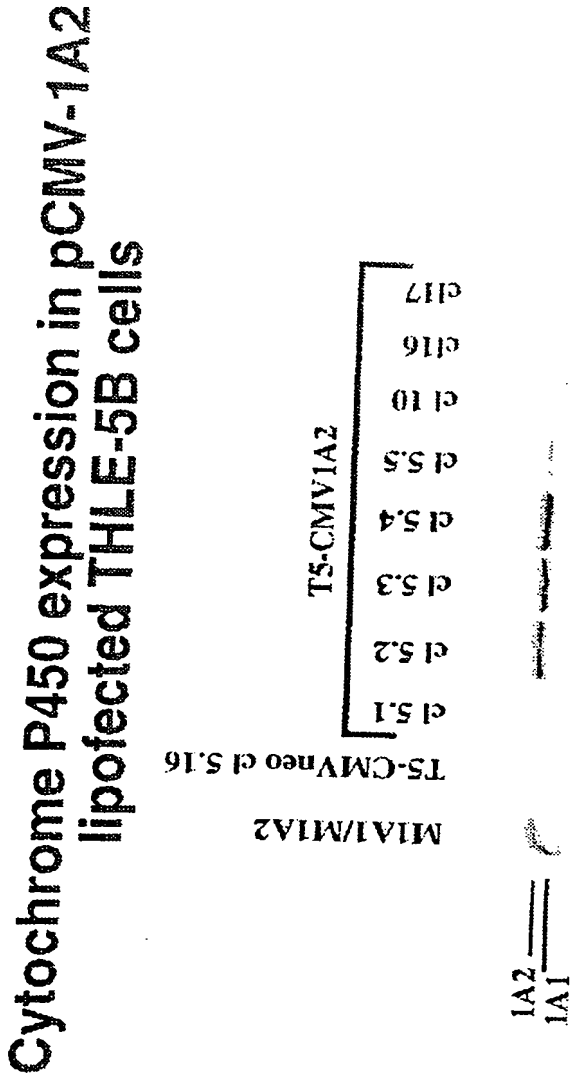
Cytochrome P450 expression in pCMV-1A2
lipofected THLE-5B cells
1A2
1A1
M1A1/M1A2
T5-CMVneo cl 5.16
T5-CMV1A2
cl 5.1
cl 5.2
cl 5.3
cl 5.4
cl 5.5
cl 10
cl16
cl17

FIG. 8

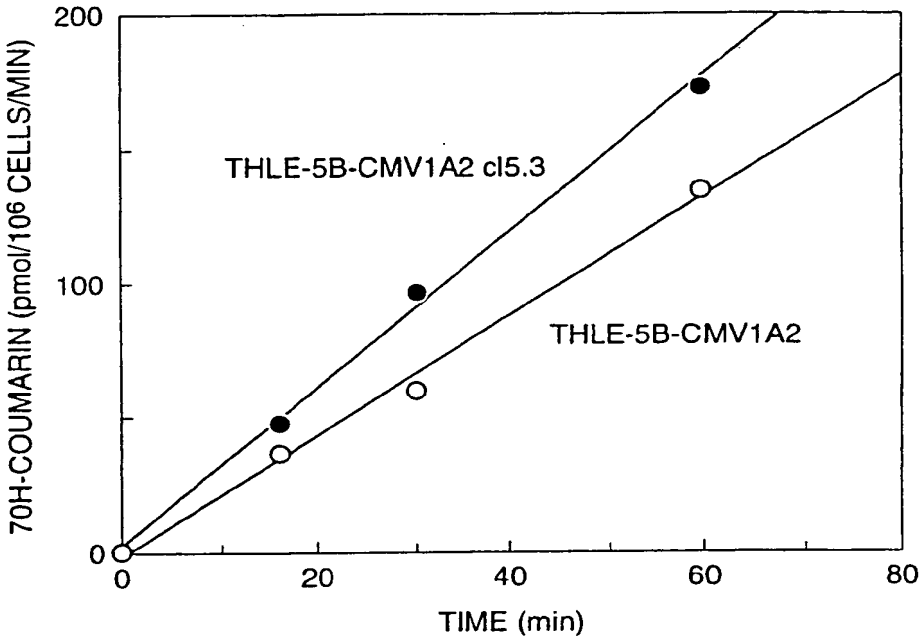
200
100
0
70H-COUMARIN (pmol/10⁶ CELLS/MIN)
THLE-5B-CMV1A2 cl5.3
THLE-5B-CMV1A2
0
20
40
60
80
TIME (min)

FIG. 9

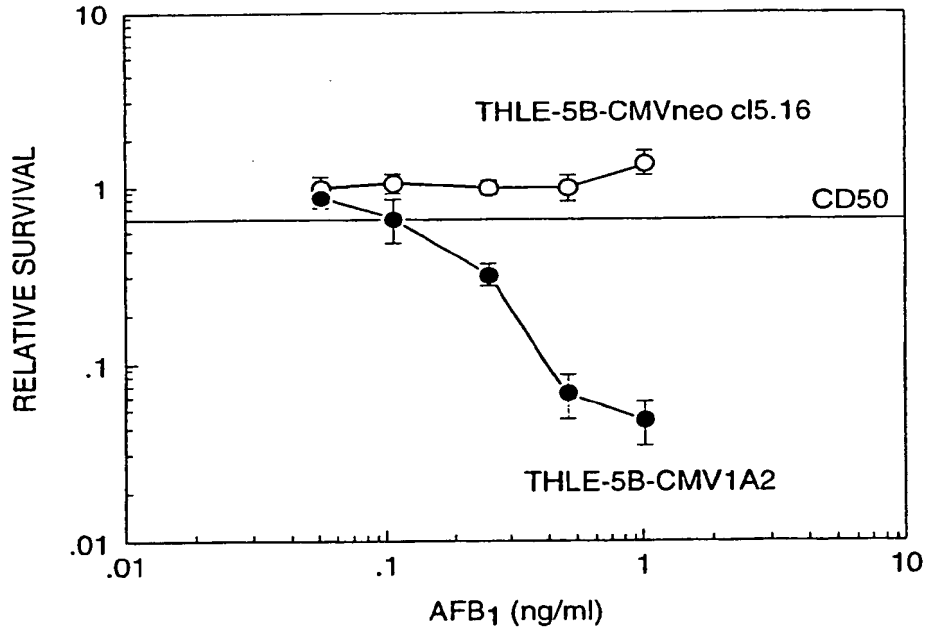
10
1
.1
.01
RELATIVE SURVIVAL
THLE-5B-CMVneo cl5.16
CD50
THLE-5B-CMV1A2
.01
.1
1
10
AFB1 (ng/ml)

FIG. 10

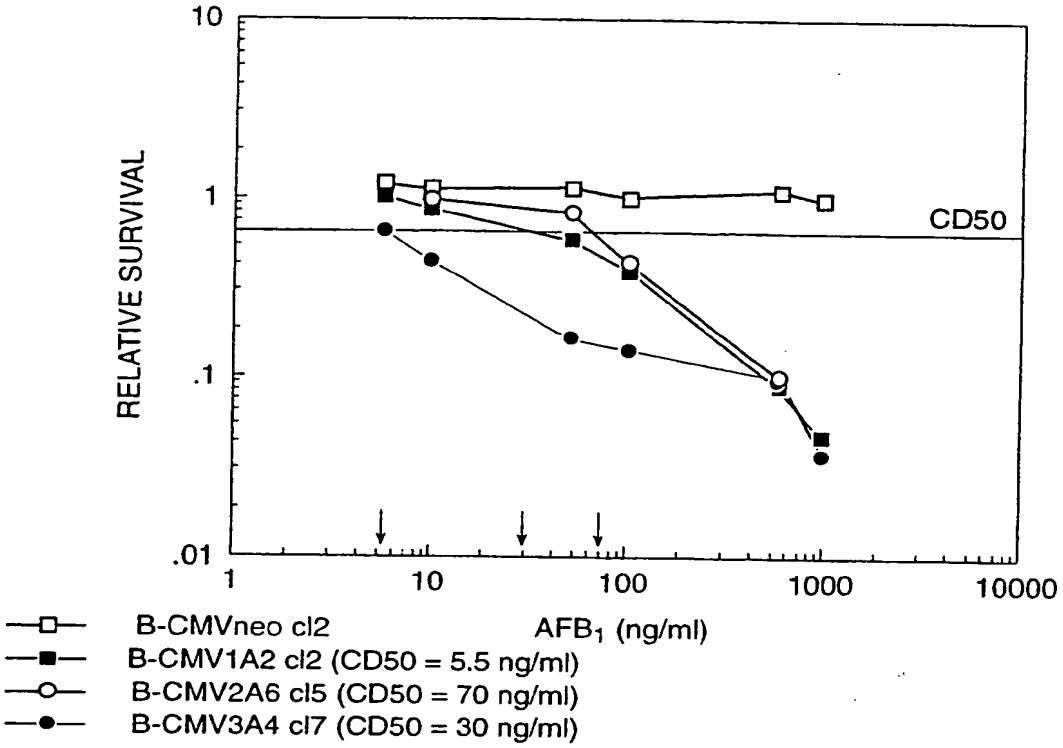
10
1
.1
.01
RELATIVE SURVIVAL
CD50
1
10
100
1000
10000
AFB1 (ng/ml)
B-CMVneo cl2
B-CMV1A2 cl2 (CD50 = 5.5 ng/ml)
B-CMV2A6 cl5 (CD50 = 70 ng/ml)
B-CMV3A4 cl7 (CD50 = 30 ng/ml)

FIG. 11

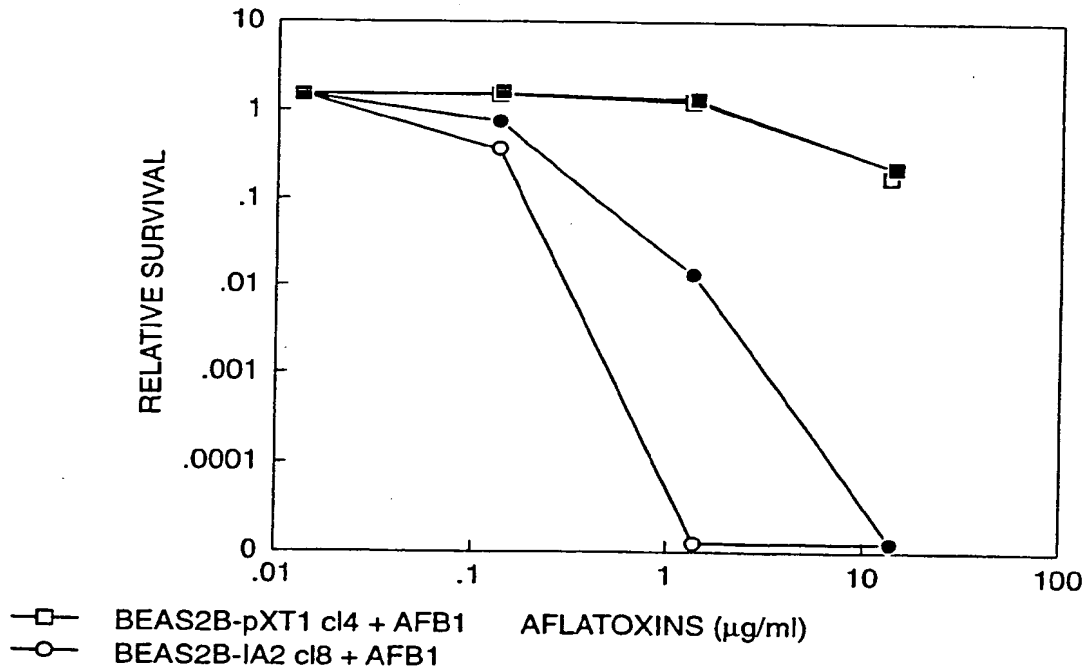
10
1
.1
.01
.001
.0001
0
RELATIVE SURVIVAL
.01
.1
1
10
100
AFLATOXINS (µg/ml)
BEAS2B-pXT1 cl4 + AFB1
BEAS2B-IA2 cl8 + AFB1
BEAS2B-pXT1 cl4 + AFG1
BEAS2B-IA2 cl8 + AFG1

FIG. 12

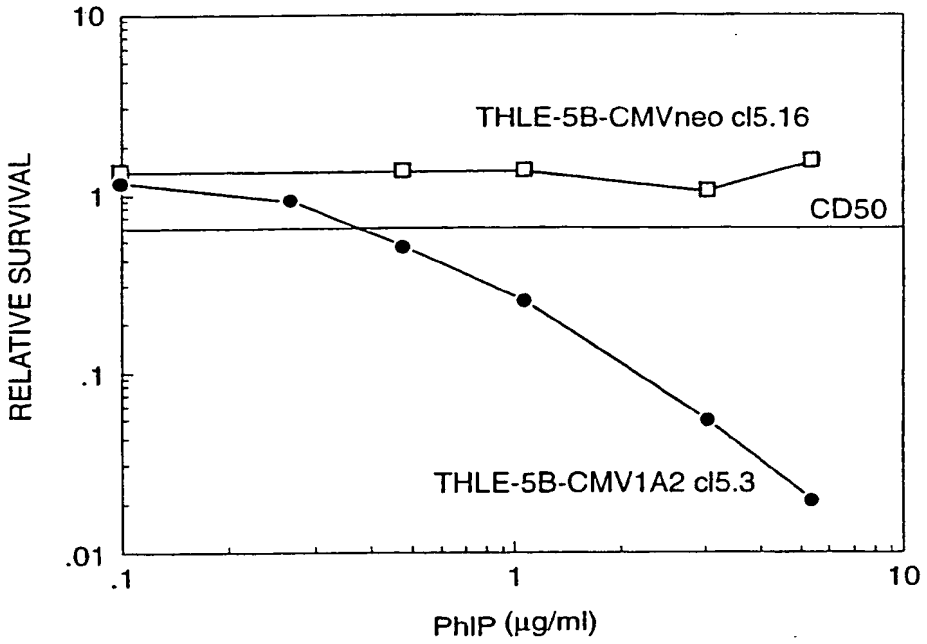
10
1
.1
.01
RELATIVE SURVIVAL
THLE-5B-CMVneo cl5.16
CD50
THLE-5B-CMV1A2 cl5.3
.1
1
10
PhIP (µg/ml)

FIG. 13

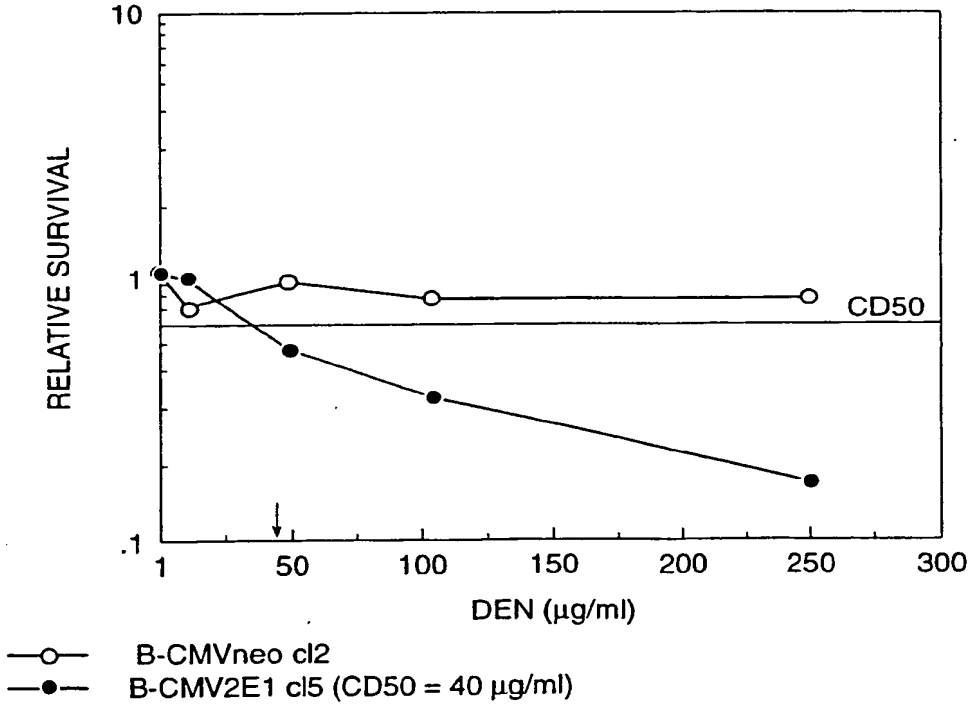
10
1
.1
RELATIVE SURVIVAL
CD50
1
50
100
150
200
250
300
DEN (µg/ml)
B-CMVneo cl2
B-CMV2E1 cl5 (CD50 = 40 µg/ml)

FIG. 14

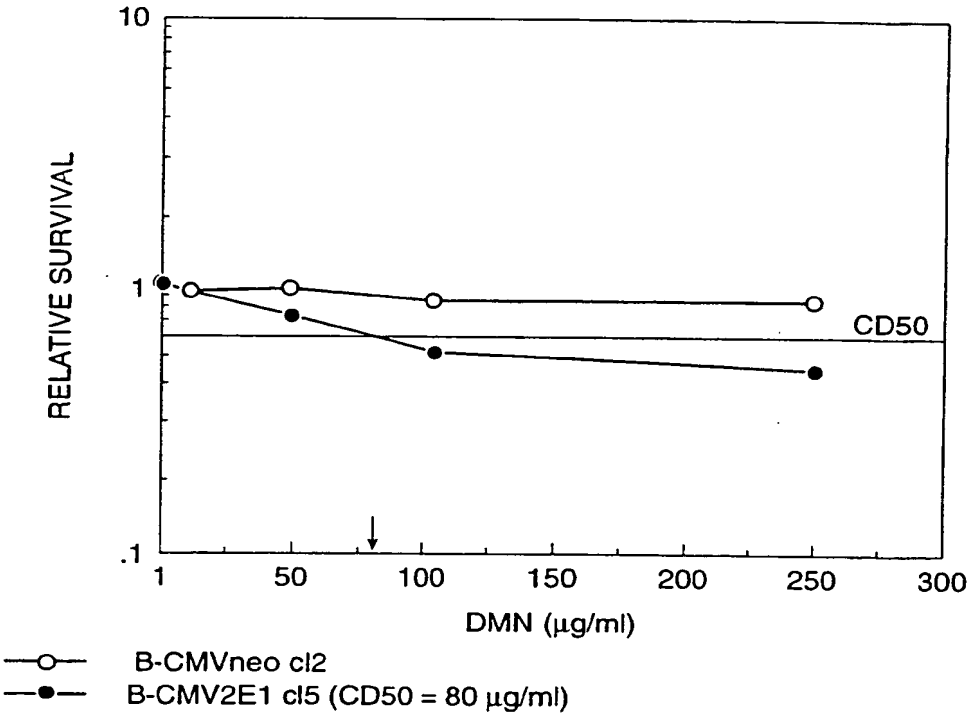
10
1
.1
RELATIVE SURVIVAL
CD50
1
50
100
150
200
250
300
DMN (µg/ml)
B-CMVneo cl2
B-CMV2E1 cl5 (CD50 = 80 µg/ml)

FIG. 15

INTERNATIONAL SEARCH REPORT

Inter nal Application No
PCT/US 94/05472

A. CLASSIFICATION OF SUBJECT MATTER
IPC 5 C12N15/53 C12N5/10 C12Q1/26 G01N33/50

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 5 C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	PROCEEDINGS OF THE AMERICAN ASSOCIATION OF CANCER RESEARCH ANNUAL MEETING vol. 33, no. 0 , March 1992 page 158 F. GONZALES ET AL 'Increased activation of promutagens in a human bronchial epithelial cell line stably expressing the human cytochrome p450 IA2' & 83RD ANNUAL MEETING OF THE AMERICAN ASSOCIATION FOR CANCER RESEARCH . MAY 20-23 1992 ;SAN DIEGO . * see abstract n0 945 * --- -/--	1-9,12, 13

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
12 September 1994	26 -09- 1994

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax (+31-70) 340-3016	Le Cornec, N

Form PCT/ISA/210 (second sheet) (July 1992)

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	PROCEEDINGS OF THE AMERICAN ASSICIATIONFOR CANCER RESEARCH vol. 34, no. 0 , March 1993 page 174 K. MACÉ ET AL 'High expression of cytochrome P-450 IA2 in SV40 T-antigen immortalized adults liver epithelial cell lines' & 84TH ANNUAL MEETING OF THE AMERICAN ASSOCIATION FOR CANCER RESEARCH, ORLANDO, USA, MAY 19-22, 1993 * see abstract 1041 *	14-22, 25,26
Y	WO,A,89 03994 (THE UNITED STATES OF AMERICA) 5 May 1989 see page 12, line 20 - page 17 see example 2	1-26
Y	MOLECULAR CARCINOGENESIS vol. 3, no. 1 , 1990 pages 5 - 8 C. L. CRESPI ET AL 'Stable expression of human cytochrome P450IA2 cDNA in a human lymphoblastoid cell line : role of the enzyme in the metabolic activation of aflatoxin B1' see the whole document	1-26
Y	CHEMICAL RESEARCH IN TOXICOLOGY vol. 4, no. 5 , October 1991 pages 566 - 572 C. L. CRESPI ET AL 'A metabolically competent human cell line expressing five cDNAs encoding procarcinogen-activating enzymes . application to mutagenicity testing' see the whole document	1-26
P,X	WO,A,93 21327 (THE GOVERNMENT OF THE UNITED STATES OF AMERICA) 28 October 1993 see the whole document	1-13
Y	WO,A,92 07085 (GENTEST CORPORATION) 30 April 1992 see the whole document	1-26
Y	US-PUBLISHED-PATENT -APPLICATION-7-377967. PUBLISHED IN NTIS GAZETTE 11-07-1989 (K. H. COLE , J.F. LECHNER, R. REDDEL, C.C HARRIS AND A.M. PFEIFER) see the whole document	1-26

-/--

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	JOURNAL OF CELLULAR BIOCHEMISTRY vol. 15D , 1991 page 140 J.F. LECHNER ET AL 'Non-tumorigenic human liver epithelial cell cultures for chemical and biological carcinogenesis investigations' & KEYSTONE SYMPOSIA ON MOLECULAR & CELLULAR BIOLOGY ; FEBRUARY 2 - MARCH 1, 1991. -----	1-26

INTERNATIONAL SEARCH REPORT

In ational application No.
PCT/US94/05472

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [ ] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. [X] Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

According to claims 14 and 21 to which refers claim 22, the human cell line should be a human adult epithelial liver cell line THLE-53 and not BEAS-2B which is an epithelial human bronchial cell line. There must be a typing error which has been taken into account when having searched the claims .

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. [ ] As all searchable claims could be searches without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

...information on patent family members

Inter nal Application No
PCT/US 94/05472

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
WO-A-8903994	05-05-89	US-A-	4885238	05-12-89
		AU-A-	2783989	23-05-89
WO-A-9321327	28-10-93	AU-B-	4281693	18-11-93
WO-A-9207085	30-04-92	AU-A-	8878191	20-05-92
		EP-A-	0555315	18-08-93
		JP-T-	6504428	26-05-94